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Genetics and Biochemistry of Carbamoyl Phosphate Biosynthesis and Its Utilization in the Pyrimidine Biosynthetic Pathway

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INTRODUCTION

Carbamoyl phosphate is a common intermediate to both the pyrimidine and the arginine biosynthetic pathways and, where it is present, to the arginine dihydrolase pathway also. A generalized version of these pathways is shown in Fig. 1. This common role of carbamoyl phosphate presents biological systems with a problem of how to control the pathways separately according to their different and varying needs for pyrimidines and arginine, pathways initiated by aspartate carbamoyl transferase (ACT) and ornithine carbamoyl transferase (OCT), respectively.

Much information has been published in the last few years on the genetics and biochemistry of carbamoyl phosphate biosynthesis and utilization in the arginine and pyrimidine pathways in organisms ranging from bacteria to mammals. This review is an attempt to summarize and, to some extent, interpret this information and brings up to date the earlier reviews by O'Donovan and Neuhard (111) and Davis (38).

Certain nucleotide phosphates will be repeatedly cited. For convenience, their names will be abbreviated in the following way: adenosine 5'-monophosphate, AMP; adenosine 5'-diphosphate, ADP; adenosine 5'-triphosphate, ATP; cytidine 5'-monophosphate, CMP; cytidine 5'-diphosphate, CDP; cytidine 5'-triphosphate, CTP; guanosine 5'-monophosphate, GMP; guanosine 5'-triphosphate, GTP; inosine 5'-monophosphate, IMP; inosine 5'-triphosphate, ITP;

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orotidine 5'-monophosphate, OMP; thymidine 5'-triphosphate, TTP; thymidine 5'-monophosphate, TMP; uridine 5'-monophosphate, UMP; uridine 5'-diphosphate, UDP; uridine 5'-triphosphate, UTP; xanthine 5'-monophosphate, XMP.

BACTERIA

Escherichia coli

There appears to be only one carbamoyl phosphate synthase (EC 2.7.2.5; CPS) in *E. coli*, specified by the *pyrA* gene, unlinked to the *pyrB* gene, which codes for ACT (EC 2.1.3.2), or indeed to any other pyrimidine gene (119). Evidence that this CPS provides carbamoyl phosphate for both the arginine and pyrimidine pathways comes from the following: (i) Mutations at *pyrA* can produce a simultaneous requirement for both arginine and pyrimidines, and these requirements are simultaneously lost on reversion (119). Mutants requiring arginine but not pyrimidines may also arise (4). (ii) Other *pyrA* mutations show uracil sensitivity (119). (iii) CPS is inhibited by UMP and other pyrimidines, activated by IMP and other purines, and also activated by L-ornithine (11, 14).

More recent work has shown that the *pyrA* locus can be subdivided into two regions, since some mutants which can have their requirements partially suppressed by high ammonium concentration all map toward one end. These mutants have high levels of ammonium-dependent CPS activity relative to glutamine-dependent CPS activity (98). This observation is consistent with the finding that CPS, which has a molecular weight of 200,000, will undergo reversible dissociation after treatment with potassium thiocyanate, giving rise to subunits of approximately 140,000 and 50,000 daltons. Neither subunit has any glutamine-dependent CPS activity, but the heavy subunit has ammonium-dependent activity and is also capable of binding all the effectors. The light subunit catalyzes the hydrolysis of glutamine (149). Both subunits are probably single polypeptides, since they exhibit a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and contain titratable sulfhydryl groups—two different groups on the heavy subunit and one on the light (12, 47, 97).

The CPS reaction requires 2 mol of ATP per mol of carbamoyl phosphate synthesized. One of these is required to provide the phosphate moiety, and the other is required to meet the reactions' energy requirements (13). Recently, using bifunctional ATP analogs, evidence has been obtained for the existence of two ATP binding sites (122). The CPS enzyme exhibits Michaelis-Menten kinetics with respect to bicarbonate and L-glutamine (K_m = 1.2 and 0.38 mM, respectively) and sigmoidal kinetics with respect to ATP which is half-maximal at 8 mM (13). The enzyme is therefore clearly allosteric. It is known to exist in a number of conformational states and also has a tendency to dimerize, giving an equilibrium between forms at 200,000 and 400,000 daltons (11, 150). However, this association-reassociation does not appear to be involved in regulation, since full catalytic and regulatory properties are retained by both the monomeric and dimeric forms (9). It has been demonstrated that one of the conformational states facilitates binding by the substrate $Mg \cdot ATP^{2-}$, and this is stabilized by the positive allosteric effectors ornithine and IMP. Another state is stabilized by the inhibitor UMP (10).

In view of the absence of the enzymes of the arginine dihydrolase system from the enteric bacteria, there appears to be no other source of carbamoyl phosphate.

ACT has been purified, and a great deal of work has been carried out on it, much of it outside the scope of this review. The enzyme is inhibited by CTP, and to some extent by other cytosine derivatives, but not by uracil compounds. It is activated by ATP, but not by guanine derivatives. It shows sigmoidal kinetics with respect to L-aspartate, half-maximal at 5.5 mM (51). Carbamoyl phosphate only gives rise to sigmoidal kinetics at very low concentrations, half-maximal at 0.2 mM (19).

Heat or mercurial treatment causes ACT to dissociate into two dissimilar subunits (50, 52, 53). The native enzyme has a molecular weight of 300,000, whereas the subunits are of 100,000 and 34,000. The larger subunits exhibit catalytic activity, but lack cooperative binding of L-aspartate and are insensitive to both CTP and ATP. They have a pH optimum of 8.5 (that for native ACT is 7.0) and K_m values of 0.06 mM for carbamoyl phosphate and 12 mM for L-aspartate. The smaller subunits, on the other hand, have no catalytic activity, but are able to bind CTP and ATP (19, 50, 51, 53). The catalytic subunits comprise three polypeptide chains of 33,000 daltons, whereas the regulatory subunits are dimers of 17,000-dalton polypeptides, with a total of six regulatory chains and six catalytic chains per molecule (154). These findings were confirmed by X-ray crystallography, which showed that the molecule has both threefold and twofold symmetry (156), and also by the discovery that ACT has six tightly bound zinc ions per molecule (134). These zinc ions are localized in the regulatory chains, are not required for binding CTP (135), do not result in a conformational change, and are thought to function by stabilizing the quaternary structure

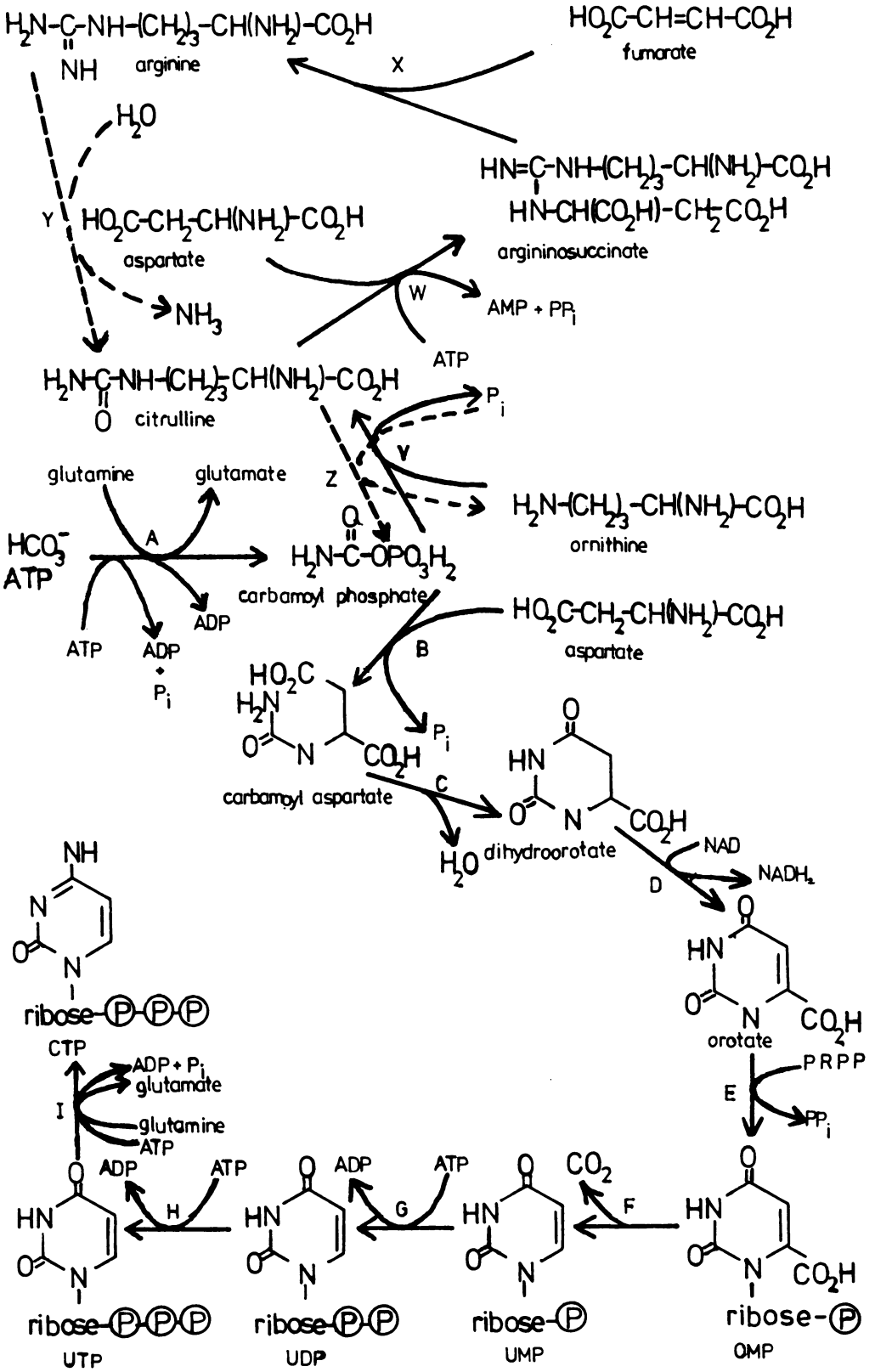

FIG. 1. *Generalized biosynthetic pathway for pyrimidines, showing relevant stages of arginine metabolism. (A) Carbamoyl phosphate synthase; (B) aspartate carbamoyl transferase; (C) dihydroorotase; (D) dihydroorotate dehydrase; (E) OMP pyrophosphorylase; (F) OMP decarboxylase; (G) UMP kinase; (H) CTP synthase; (V) ornithine carbamoyl transferase (anabolic); (W) argininosuccinate synthase; (X) argininosuccinase; (Y) arginine deiminase; (Z) citrullinase (catabolic OCT).*

of the enzyme (27, 104). The three-dimensional structure of the enzyme appears to consist of two catalytic trimers separated by three regulatory dimers (27, 155). In the center of the molecule is a large cavity which appears to be accessible for active sites (44, 153). Access to this cavity seems to be via six channels near the regulatory chains, a finding which has led to the speculation that the regulatory mechanism may involve modulation of access of substrates through these channels (153).

The *argF* and *argI* genes specify two OCT enzymes, and *pyrC* specifies dihydroorotase (DHO). The *argI* gene is next to the ACT structural gene, *pyrB*. Either *argF* or *argI* may have a catabolic role (citrullinase activity), whereas the other catalyzes citrulline biosynthesis. Knight and Jones (79) have recently demonstrated a regulatory link between the arginine and pyrimidine biosynthetic pathways by showing that OCT is activated by the pyrimidine orotate except at low carbamoyl phosphate and ornithine concentrations, under which conditions orotate becomes inhibitory.

Salmonella typhimurium

The organization of CPS and ACT in *Salmonella* is very similar to that found in *E. coli*. There is one CPS which functions in both biosynthetic pathways. Mutants in *pyrA* have a similar range of phenotypes (2, 42, 141, 160), and CPS activity is modified by the same range of compounds. Furthermore, it is strongly activated by 5-phosphoribosyl-1-pyrophosphate (PRPP) (1, 3) and is subject to repression by arginine and a cytosine compound.

CPS has a molecular weight of 150,000, but in the presence of ornithine exists as a tetramer of molecular weight 580,000. Treatment with potassium thiocyanate leads to reversible dissociation to two dissimilar polypeptides of 110,000 and 45,000 daltons. As with *E. coli*, the larger subunit has been shown to catalyze ammonium- but not glutamine-dependent CPS activity, whereas the smaller subunit restores the full activity. Both polypeptides are encoded at the *pyrA* locus, presumably by two adjacent genes (3).

As with CPS from *E. coli*, that from *S. typhimurium* shows sigmoidal kinetics with magnesium ATP, being half-maximal at 1.4 mM. The heavy subunit also exhibits sigmoidal kinetics, half-maximal at 0.3 mM (3).

ACT activity is encoded at the *pyrB* locus, which, as with *E. coli*, is unlinked to *pyrA* or any other pyrimidine gene (160). ACT is inhibited by CTP, but more strongly than the *E. coli* enzyme, being maximal at 95% instead of 75%, and it is also activated by ATP (110). Mutants with reduced sensitivity of ACT to CTP have been isolated, mainly by resistance to 5-fluorouracil or 5-fluorouridine, and these cotransduce with *pyrB* mutants (109). However, the gene encoding the regulatory polypeptide appears to be distal to *pyrB*, since nonsense and frameshift mutants in the *pyrB* locus still have regulatory subunits but at a low level (142).

The native ACT enzyme of 300,000 daltons undergoes dissociation to catalytic and regulatory subunits in the same way as ACT from *E. coli*. Catalytic subunits and the native enzyme exhibit very similar kinetics to those in *E. coli*. In fact, both types of hybrid between *S. typhimurium* and *E. coli*, regulatory plus catalytic subunits and vice versa, have been produced, and both are fully functional, suggesting that intersubunit contact regions have been conserved during evolution (110).

The *pyrC* gene specifies DHO. *argI* is the structural gene for OCT and is adjacent to the ACT structural gene *pyrB*.

Bacillus subtilis

It was originally thought that there was only one CPS enzyme in *B. subtilis*, as with the two previous organisms. This was because selection for arginine and/or pyrimidine auxotrophs gave rise to double auxotrophs lacking any detectable CPS activity (64). However, more recently, both arginine- and uracil-sensitive mutants have been isolated. The former class cotransforms with two pyrimidine auxotrophs, whereas the latter does not (120, 121). It therefore would appear that there are two CPS enzymes in *B. subtilis* and that the carbamoyl phosphate synthesized is not efficiently channeled, since strains lacking one CPS are able to utilize carbamoyl phosphate from the other pathway.

Another important difference between *B. subtilis* and the bacteria considered earlier is that in this case all the known pyrimidine genes are linked. Also, some ACT^- mutants are also lacking CPS and DHO (EC 3.5.2.3). CPS, ACT, and DHO show similar but not identical molecular weights on a sucrose density gradient, with ACT slightly lighter and DHO slightly heavier than CPS, all three approximating 130,000 (121). These may of course be fragments resulting from dissociation or proteolysis of a larger, multifunctional complex.

Some properties of the CPS activity have been described, but since they appear to refer to the combined activities of the two CPS enzymes, the arginine-sensitive CPS and the pyrimidine-sensitive CPS, their usefulness is somewhat limited. Activation is achieved by PRPP, L-ornithine, and *N*-acetylglutamate, whereas inhibition is by UTP, with CTP and IMP being ineffective. Ki-

netics with respect to all three substrates appear to be hyperbolic (64, 120).

ACT is completely insensitive to all pyrimidine nucleotides, but is stimulated by a number of anions. It shows Michaelis-Menten kinetics for both substrates, giving K_m values of 5.7 mM for L-aspartate and 0.5 mM for carbamoyl phosphate, and has a pH optimum of 8.5 (21, 105). It has been purified, presumably as a subunit, and has a molecular weight of 102,000. SDS-polyacrylamide gel electrophoresis suggests a trimeric protein of 33,500-dalton polypeptides. The properties of these catalytic subunits are somewhat similar to the ACT catalytic chains from *E. coli* and *S. typhimurium*, including its amino acid composition. However, these similarities are not sufficient to permit cross-reaction between antibodies to *B. subtilis* ACT and ACT catalytic chains from *E. coli* (21).

There is some doubt as to whether *B. subtilis* has the enzymes of the arginine dihydrolase system. It does possess a carbamoyl phosphokinase (EC 2.7.2.2; CPK) and two OCTs (EC 2.1.3.3), one of which may have a citrullinase function in vivo (16). However, recent work by Broman et al. (23) showed that in addition to these enzymes, the related organism *Bacillus licheniformis* also has arginine deiminase (EC 3.5.3.6). All three enzymes of the dihydrolase system are inducible, are only present in high concentrations under anaerobic conditions, and seem to function as an energy source, since ATP is generated in the CPK reaction. If these enzymes are also present in *B. subtilis*, it seems likely that any carbamoyl phosphate produced under anaerobic conditions would have a transitory existence and hence be unable to be utilized by the pyrimidine biosynthetic pathway.

Lactobacillus leichmannii

This organism has an absolute requirement for arginine, which it appears to utilize for energy and for the biosynthesis of pyrimidines and purines. It seems to lack any CPS enzymes, but possesses the arginine dihydrolase system.

Tracer studies have shown that the guanidino carbon atom of arginine, and not the carbon from carbon dioxide, is utilized as a pyrimidine precursor. Arginine deiminase is inhibited by TMP, but not by cytidine or uridine nucleotides, and also is inhibited by purine nucleotides (62). Because of these observations, the pathway for pyrimidine biosynthesis shown in Fig. 2 was proposed for this organism.

ACT and DHO are both subject to repression by uracil. However, ACT is not inhibited by any pyrimidine nucleotide. It has a pH optimum of 8.5 and exhibits hyperbolic kinetics with L-aspartate, the K_m being 30 mM. Its dependence on carbamoyl phosphate showed substrate inhibition (62).

Streptococcus faecalis

S. faecalis, like the previous organism, is a lactic acid bacterium, deriving its energy from the fermentation of sugars. Like *L. leichmannii*, it has an arginine requirement and is unable to convert citrulline to arginine. It possesses the arginine dihydrolase system, and although its CPK can synthesize carbamoyl phosphate, this particular enzyme greatly favors the reverse reaction leading to ATP production. There is no evidence of any CPS activity (69, 70).

In common with ACT from *B. subtilis* and *L. leichmannii*, ACT from *S. faecalis* is uninhibited by pyrimidine nucleotides. Furthermore, it has a pH optimum of 8.5. However, its kinetics are complex. At low buffer strengths, very large activation has been observed with a number of anions. Under these conditions, the kinetics deviate from Michaelis-Menten and are further complicated by substrate inhibition. At high acetate concentrations, hyperbolic plots of substrate dependence were obtained, showing a 50-fold increase in affinity for carbamoyl phosphate. The existence of an allosteric activator site distinct from the substrate binding site has been proposed, with the suggestion that L-aspartate may be the physiological activator (26, 69, 123).

S. faecalis ACT has a molecular weight of 130,000 and, after electrophoresis of SDS-treated extracts, one band corresponding to 32,500 was observed. This suggested that ACT is tetrameric. The *S. faecalis* ACT showed no cross-reaction with antibodies to *E. coli* ACT.

Pseudomonas fluorescens

The fluorescent pseudomonads (*P. fluorescens, P. aeruginosa,* and *P. putida*) all possess the arginine dihydrolase system. This functions to provide energy for motility under anaerobic conditions. Although arginine dihydrolase is thought to be constitutively synthesized, it appears to be inactive in the presence of oxygen (140). As appears likely with the genus *Bacillus*, when the arginine dihydrolase enzymes function, carbamoyl phosphate probably only exists in a transitory state and is therefore unlikely to act as a precursor for pyrimidine biosynthesis.

This organism possesses CPS activity, although it is not known whether more than one enzyme is involved. However, if there is a CPS-ACT complex, it is very unstable, since during the purification procedure for ACT, CPS activity was continuously lost (6).

ACT activity is inhibited by pyrimidine nucleotides and also by purines. By far the most

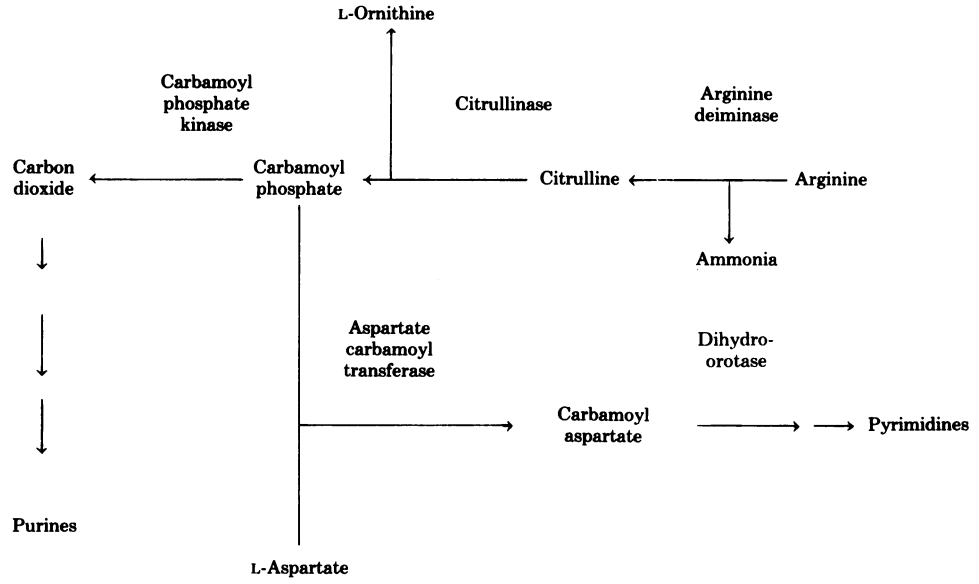

FIG. 2. *Pyrimidine biosynthesis from arginine via the arginine deiminase system.*

potent inhibitor is inorganic pyrophosphate, inhibition being competitive with carbamoyl phosphate (6, 18, 105). In common with ACT from many other bacteria, the pH optimum in this case is also 8.5. Both carbamoyl phosphate and L-aspartate give hyperbolic substrate concentration curves, with K_m values of 0.014 and 2.75 mM, respectively. However, in crude extracts, UTP converts the carbamoyl phosphate dependence curve to a sigmoidal one, but since this is not the case in purified preparations, its significance is questionable (6).

ACT has a molecular weight of 360,000. Electrophoresis of SDS-treated purified extracts demonstrated that it comprises two subunits of 180,000 daltons (6).

Pseudomonas aeruginosa

A number of ACT$^-$ pyrimidine auxotrophs have been isolated, and these all map at the *pyrB* locus, which is unlinked to all other known pyrimidine loci. As with *P. fluorescens*, ACT from *P. aeruginosa* is inhibited by UTP, CTP, ATP, and GTP. The activity of the ACT enzyme also has a similar pH optimum (63). Its molecular weight is very similar to that of the *P. fluorescens* enzyme (18).

There appears to be only one enzyme responsible for glutamine-dependent CPS activity, and this supplies carbamoyl phosphate to both the arginine and pyrimidine pathways. Single mutants lacking this activity require both arginine and uracil for growth. Such mutants map at the *car* locus, which is unlinked to any *arg* or *pyr* locus. There is no evidence for separate genes for ammonium-dependent CPS and glutaminase, suggesting that CPS comprises only one type of polypeptide chain (55).

Pseudomonas putida

ACT from *P. putida* is much less powerfully inhibited by CTP and UTP than that from the other pseudomonads, and purines have little or no effect on its activity. As with ACT from *P. fluorescens*, inorganic pyrophosphate is by far the most potent inhibitor. The kinetics of ACT in *P. putida* closely resemble those in *P. fluorescens* (30).

Aeromonas salmonicida

Pyrimidines from this fish pathogen are very inefficiently labeled when [^{14}C]bicarbonate is administered. In contrast, [^{14}C]arginine, -citrulline, -carbamoyl aspartate, and -orotate all give rise to extensive labeling in pyrimidine nucleotides. A pathway similar to that observed in *L. leichmannii*, with the initial step in the pyrimidine pathway being catalyzed by arginine deiminase, has been proposed. Like the enzyme from *L. leichmannii*, this arginine deiminase is inhibited by purine nucleotides but not by cytidine or uridine nucleotides. Unlike the enzyme in that organism, it is not inhibited by TMP (136).

Citrobacter freundii

Bethel and Jones (18) showed that ACT from this bacterium, and also from *Proteus vulgaris*,

has two molecular-weight forms. Gel filtration gave molecular weights of 300,000 and 100,000, similar in size to native ACT and the catalytic ACT subunit of *E. coli*. The two forms varied in relative amounts according to the stage of growth at which the cells were harvested. The larger form could be converted to the smaller form in vitro, but not vice versa (28). In the light of recent observations in a number of organisms, it seems probable that the smaller ACT form is a product of proteolysis of the larger (95, 124).

The nucleotides GTP, UTP, and CTP and inorganic pyrophosphate inhibit both forms of ACT. ATP stimulates the larger form, but has no effect on the smaller. Both forms of the enzyme show Michaelis-Menten kinetics with both carbamoyl phosphate and L-aspartate (28).

Serratia marcescens

There is some controversy about the properties of the ACT of this organism. Neumann and Jones (105) claimed that ACT is inhibited by UMP and CMP. Using a different strain, J. R. Wild (Ph.D. thesis, University of California, Riverside, 1971) reported that ACT is activated by ATP, CTP, and CDP, but that AMP, CMP, and guanosine and uridine nucleotide phosphates have no effect. He was unable to demonstrate inhibition by any nucleotide. Both reports agree that the enzyme can be desensitized by heat treatment, and Wild demonstrated this effect also with parachloromercuribenzoate.

Native ACT has a molecular weight of approximately 300,000, whereas the desensitized ACT is a protein of 100,000. In this respect, the situation is similar to that found in *E. coli* and *Salmonella typhimurium* (18; Wild, Ph.D. thesis, 1971).

Wild (Ph.D. thesis, 1971) reported the existence of a CPS enzyme in *Serratia marcescens*, but referred to difficulties in its assay.

Halobacterium cutirubrum

This organism is an obligate halophile, and not surprisingly its ACT requires a high salt concentration, having maximum stability and activity in the range 3.5 to 4.0 M sodium chloride. It is subject to feedback inhibition by CTP, has a pH optimum of 7.5, and exhibits hyperbolic kinetics with carbamoyl phosphate (K_m = 2.7 mM at 3.5 M NaCl). With fresh extracts, L-aspartate gives sigmoidal kinetics (87, 106), but after 2 h of storage becomes hyperbolic (K_m = 10 to 15 mM).

ACT has a molecular weight of 160,000. Treatment with polyethylene glycol leads to dissociation to subunits of 34,000 daltons which are insensitive to CTP (106).

FUNGI

Saccharomyces cerevisiae (Yeast)

There are two CPS enzymes in yeast. The pyrimidine-specific one (CPSpyr) and ACT are both encoded by the *ura2* locus, whereas the arginine-specific enzyme (CPSarg) is specified by two separate loci, *cpa1* and *cpa2*. CPS^- mutants in *ura2* result in arginine sensitivity of carbamoyl phosphate biosynthesis and prototrophy, whereas mutants in either *cpa1* or *cpa2* are uracil sensitive. Auxotrophy to either pyrimidines or arginine does not arise separately, showing that carbamoyl phosphate produced in one pathway is available for use in the other, as has been seen in *B. subtilis* (85). The *arg3* gene codes for OCT, and *ura4* codes for DHO.

The *ura2* locus has been extensively studied. CPS^- ACT^+, CPS^+ ACT^-, and CPS^- ACT^- mutants have all been isolated, the first two types complementing well with each other. CPS^+ ACT^- mutants are confined to one end of the locus, and the other two types map throughout the remainder. No nonsense CPS^- ACT^- mutants map in the ACT region, suggesting a single messenger ribonucleic acid from CPS to ACT (40).

Both CPSpyr and ACT are powerfully inhibited by UTP. Furthermore, CPSpyr is also slightly inhibited by UDP, UMP, CTP, CMP, GTP, TTP, and ATP (84, 85). In common with *E. coli*, heating causes ACT to become progressively insensitive to feedback inhibition before loss of catalytic activity (72, 74). Inhibition of ACT is also progressively lost during repression, although this is likely to be a destabilization effect as a result of the action of uracil (73). Subsequent studies on CPS activity have shown that whereas IMP has no effect, the purine XMP is a potent inhibitor (8).

Feedback-resistant mutants have been isolated from among 5-fluorouracil-resistant mutants. In all cases both CPSpyr and ACT activities were insensitive to UTP. These feedbackless mutants have been shown to map at the *ura2* locus (84).

A CPSpyr-ACT multienzyme complex has been purified from yeast, and its molecular weight is estimated at 800,000 (originally reported as 600,000). In the absence of UTP, the molecular weight was found to be approximately halved to 380,000, and the ACT activity in this form had reduced feedback sensitivity (88, 91). Passage through diethylaminoethyl-Sephadex and heat treatment caused loss of CPS activity together with loss of feedback sensitivity of ACT, in a form with a molecular weight of 140,000 (88, 89). Alternatively, omission of UTP,

magnesium ions, and L-glutamine from the sucrose density gradient led to the production of an additional peak of CPS activity at a molecular weight of 250,000 (91). Tempting though it might be to speculate that the complex comprises two CPS subunits of 250,000 daltons and two ACT subunits of 140,000 daltons, it is worth remembering that at no time have both species been simultaneously observed as a result of dissociation of the complex.

The ACT molecule of 140,000 daltons has been shown to comprise polypeptides of 21,000 daltons by SDS-gel electrophoresis, suggesting that it is a hexamer (7).

Double mutants, in both CPSpyr and CPSarg, are necessary to generate auxotrophy, suggesting that carbamoyl phosphate is not efficiently channeled into the two pathways. However, $CPSarg^-$ mutants grow much faster in the presence of arginine, whereas $CPSpyr^-$ mutants grow at wild-type rate even in minimal medium. Possibly carbamoyl phosphate is more readily transferred from the arginine to the pyrimidine pathway than vice versa, although differential rates of synthesis in the two pathways is a more likely explanation. Paradoxically, in vitro the multienzyme complex does seem to channel carbamoyl phosphate, since the compound in free solution will not exchange with that synthesized by the enzyme (90).

The kinetics of both CPSpyr and ACT activities have been examined. Both give hyperbolic curves with all substrates. K_m values for ACT are 30 mM for L-aspartate and 4 mM for carbamoyl phosphate in the native multienzyme complex and 43 and 3.4 mM, respectively, in the 140,000-dalton subunit (7, 72). The K_m values for CPSpyr are 3.5 mM (ATP), 0.2 mM (L-glutamine), and 7 mM (bicarbonate) (8). The pH optima are at 7.4 for CPSpyr and in the range of 8.0 to 8.7 for ACT.

Aspergillus nidulans

A number of pyrimidine auxotrophs have been isolated and analyzed. $CPS^- ACT^+$, $CPS^+ ACT^-$, and $CPS^- ACT^-$ mutants all appear to map at the *pyrA* locus, not closely linked to any other known pyrimidine locus, including that specifying DHO, the *pyrD* locus. Within *pyrA*, the $CPS^- ACT^+$ mutants complement the $CPS^+ ACT^-$ mutants, and some mutants within the latter group complement each other (117).

There appear to be two CPS enzymes, as with other fungi so far examined. Auxotrophy of $CPS^- ACT^+$ alleles was found to be suppressed by a mutant with low OCT activity. This suggested an organization similar to that found in the fungi to be discussed later, in which carbamoyl phosphate will only overflow from one pathway to the other when the first pathway is blocked at the enzyme which utilizes carbamoyl phosphate as its substrate.

A number of mutants of *Aspergillus* resistant to 5-fluorouracil were isolated, and some were found to be very closely linked to the CPSpyr-ACT locus, *pyrA*. These were feedback-insensitive mutants, similar to those found at the homologous locus of *Saccharomyces cerevisiae*. Another class of resistant mutants was found to be due to a mutation on a different linkage group which resulted in greatly reduced sensitivity of ACT to repression by uridine (118).

Neurospora crassa

In *Neurospora* there are again two CPS enzymes. The CPSarg is specified by two genes, *arg-2* on linkage group IV and *arg-3* on linkage group I. Mutations at either gene result in an arginine requirement. The CPSpyr enzyme is encoded at the *pyr-3* locus on linkage group IV, very close to the *arg-2* locus (130). Mutations at *pyr-3* result in a requirement for a pyrimidine compound (37). A mutation in the gene for OCT, *arg-12* on linkage group II, will suppress the pyrimidine requirement caused by a $CPSpyr^-$ mutation at *pyr-3*, whereas ACT^- mutants at *pyr-3* will suppress the arginine requirement brought about by a $CPSarg^-$ mutation at *arg-2* or *arg-3* (34, 36, 39, 132). Wild-type extracts exhibit two CPS peaks on gel filtration, one of which is absent in $CPSarg^-$ mutants and the other from $CPSpyr^-$ mutants (159).

Mutants at the *pyr-3* gene are of all three possible enzymatic types, $CPS^+ ACT^-$, $CPS^- ACT^+$, and $CPS^- ACT^-$. Due to the very low fertility of *pyr-3* interallelic crosses, reliable high-resolution data have been very difficult to obtain. However, by analysis of interallelic complementation and reversion data, a number of polar (frameshift and nonsense) alleles were identified. These were used to construct a linear map of the locus (Fig. 3) based upon the assumption that if a nonpolar allele complements with a polar one, then the nonpolar allele must be translationally proximal to the polar one (125–127; A. J. Makoff, Ph.D. thesis, Leeds University, Leeds, England, 1976). Enzymatic analysis of the *pyr-3* polar alleles showed them to be of two types, $CPS^+ ACT^-$ and $CPS^- ACT^-$, but not $CPS^- ACT^+$. Of the nonpolar alleles, the $CPS^+ ACT^-$ ones were at one end of the locus and the $CPS^- ACT^+$ were distributed toward the other. The $CPS^- ACT^-$ mutants were scattered throughout at least a large part of the locus. The mutants therefore define a proximal CPSpyr-specific region and a distal ACT-specific

region, the direction of translation being from the CPS- to the ACT-specific ends (128). This model predicts that the CPS^- ACT^- polar mutants might partially revert to CPS^- ACT^+, but never to CPS^+ ACT^-, and this was found to be the case (129).

Both CPSpyr and ACT are subject to coordinate repression by uridine, whereas the enzyme controlling the next step in the pyrimidine pathway, DHO, is unaffected. CPSpyr is subject to feedback inhibition by UTP, and to a lesser extent by UMP. Uridine, CTP, CMP, and cytidine have no effect, whereas ATP opposes inhibition by UTP (159). The feedback binding site appears to be on the same polypeptide chain as the CPSpyr active site, in view of the lack of a feedback-specific region separable from the CPS-specific region (93).

The CPSpyr activity has a pH optimum of 7.5, but no kinetic data are available. It is highly unstable both in the cold and at moderately warm temperatures, the optimum stability being at 15°C (159). Williams and Davis (159) claimed that UTP stabilized CPSpyr at 0°C and had no effect at 25°C, whereas L-glutamine stabilized at 25°C but not at 0°C. In our hands, neither UTP nor L-glutamine had any significant effect on cold- or warm-mediated inactivation (95).

The CPSarg enzyme comprises two polypeptides, being encoded at two unlinked loci, *arg-2* and *arg-3*. The *arg-2* gene product appears to be a glutaminase, whereas *arg-3* specifies an enzyme with ammonium-dependent CPS activity (37). This situation resembles that found in *E. coli* and *S. typhimurium*. Although the CPSpyr-ACT complex is oligomeric, CPSpyr activity appears to comprise only one polypeptide type. This is strongly suggested by the finding that, of the many *pyr-3* alleles examined, none with little or no glutamine-dependent CPSpyr activity owed their mutation to a loss of the glutamine binding site (94).

Another complication is provided by the fact that the *arg-2* locus is adjacent to the translationally distal end of *pyr-3*. There are four lines of evidence to show that this close proximity has no functional significance: (i) Studies by Cybis and Davis (32) showed that the *arg-2* gene product is subject to powerful repression by arginine. (ii) No *pyr-3* CPS^- ACT^- polar mutant has an arginine requirement (94). (iii) Mutants at *arg-2* have normal glutamine-dependent CPSpyr activities (93). (iv) There are two chromosomal rearrangements, in strains S1229 and S4342, involving breakpoints between *arg-2* and *pyr-3* which inactivate neither gene (15, 101).

There have been conflicting reports on the properties of the ACT activity of *Neurospora*. Donachie (41) claimed a pH optimum of 9.5, but reported variations in a number of properties of the enzyme, depending on the buffering system used. In phosphate or tris(hydroxymethyl)-aminomethane (Tris) buffer, substrate inhibition was observed. Under conditions of inhibiting L-aspartate concentrations, uridine derivatives, but not cytidine or CTP, were inhibitory. Glycine reversed this inhibition and actually activated the enzyme. In glycine buffers, no substrate or feedback inhibition was observed, and hyperbolic kinetics were obtained, with K_m values for L-aspartate and carbamoyl phosphate being 7 and 0.7 mM, respectively. Using Tris

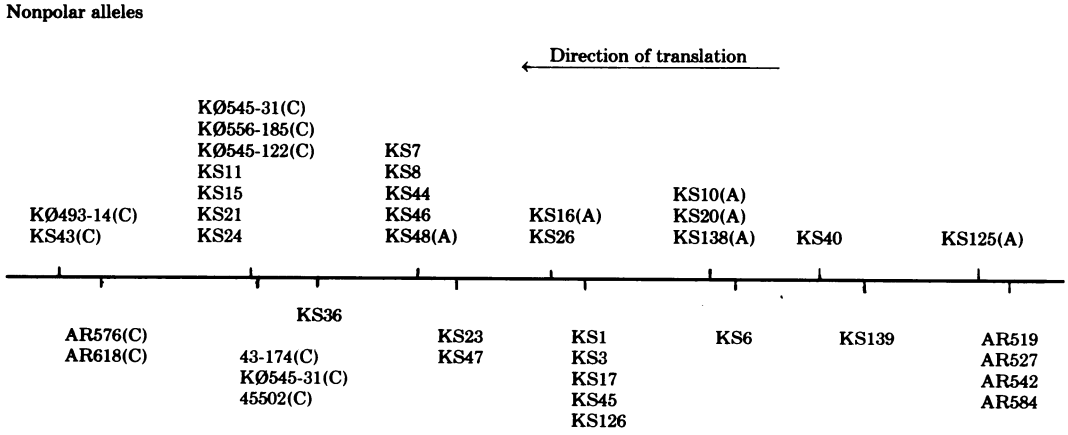

FIG. 3. *Fine-structure map of the pyr-3 locus of Neurospora. Mutants marked (A) have ACT activity but not CPSpyr activity. Mutants marked (C) have CPSpyr but not ACT activity. Unmarked mutants have neither activity.*

buffer, Hill and Woodward (58) claimed a pH optimum of 8.6, but this must be treated with suspicion since they appear to have assayed ACT at a pH outside the range of Tris buffering capacity. Davis (33) claimed a pH optimum of 9.1, using Tris-acetate and glycine-sodium hydroxide buffers, and there was some evidence of activation on changing to the glycine-containing buffer. He also claimed a K_m for L-aspartate of 5 mM, although his Lineweaver-Burk plot showed many departures from linearity. In disagreement with Donachie, he produced data to show that under a variety of conditions, no pyrimidine had any effect on ACT (159).

CPSpyr and ACT have been copurified, but not to homogeneity. Estimates of the molecular weight of the complex varied according to the method used. Using thyroglobulin, urease, and catalase markers, gel filtration gave a value of 650,000. Sedimentation on sucrose density gradients gave a value of 630,000 in the absence of UTP or 380,000 in its presence, using a catalase marker on both occasions. This has been interpreted as UTP-mediated dissociation, but this seems unlikely since the elution volume of the complex on gel filtration is unaffected by the presence or absence of UTP. The fraction of total protein undergoing this change in sedimentation was approximately 50%, and this was used to give an estimate of purity of the preparation. However, no reports of polyacrylamide gel electrophoresis were given as an alternative estimate of purity (157). More recent attempts at purification of the enzyme complex have been unsuccessful, due largely to the extreme instability of the CPSpyr, even under conditions which had been reported to stabilize it. Moreover, ACT was found in two molecular-weight forms in addition to its native form of 650,000. A high-molecular-weight form, in excess of 2,000,000, also exhibiting CPSpyr activity, occurred, and was probably an aggregate of the enzyme complex. A low-molecular-weight form at 100,000 was also found, also posessing ACT activity but lacking CPSpyr activity. This latter form was shown to be a fragment of the native complex arising by endopeptidase attack on the native form when purification had removed the endopeptidase inhibitor (95). This 100,000-dalton fragment differs from the native ACT in its affinity for L-aspartate, which is higher, and in its activation by glycine (92). Its similarity to the ACT described by Donachie probably explains the discrepancy between the data on ACT already discussed.

Apart from the above forms of the enzyme obtainable from *pyr-3*$^+$ strains, a CPSpyr fragment of 180,000 daltons has now been identified in the *pyr-3* (CPS$^+$ ACT$^-$) frameshift allele 43-174. In this mutant, the CPS region of the gene is translated, probably plus also a noncomplementing proximal part of the ACT region (this allele does not complement with any other CPS$^+$ ACT$^-$ allele) (A. J. Makoff, F. P. Buxton, and A. Radford, manuscript in preparation). The types and sizes of the fragments and the size of the native enzyme have permitted these authors to construct a model of the subunit structure of the native enzyme, in which they postulate the existence either of a hexamer of double-headed polypeptides with 3× associations of ACT and 2× associations of CPS or of two ACT trimers joined by three CPS dimers. The ACT active site in each of these models is a property of the association of three ACT domains, but the CPS activity does not in wild type require CPS-CPS contact, although it does for complementation between different CPS$^-$ alleles.

Channeling of the carbamoyl phosphate in the two pathways seems quite tight in *Neurospora*, since a single mutational event in either CPS results in auxotrophy for one or other end product. Radioactive labeling has revealed the presence of two discrete carbamoyl phosphate pools (158). Histochemical studies showed that arginine biosynthetic enzymes including CPSarg and OCT are localized in the mitochondria, whereas ACT and CPSpyr are found mainly in the nuclei (17). It therefore appears that the observed channeling of carbamoyl phosphate is the combined result of both physical separation and the existence of the CPSpyr-ACT multienzyme complex.

Coprinus radiatus

As with *N. crassa* and *S. cerevisiae*, there are two CPS enzymes, CPSarg, specified by *Arg-1* and *arg-5*, and CPSpyr, specified by *ur-1*, which also specifies ACT. The *ur-1* locus has CPS$^+$ ACT$^-$ alleles all mapping at one end, whereas the CPS$^-$ ACT$^+$ and CPS$^-$ ACT$^-$ map throughout the remainder of the locus. As in *Neurospora* and *Aspergillus*, but unlike *Saccharomyces*, a single mutation of either CPS enzyme results in auxotrophy for one or other end product, and carbamoyl phosphate can only be transferred from one pathway to the other when the next step in the donating pathway is blocked. Thus an OCT$^-$ mutation at the *arg-6* locus will suppress the pyrimidine requirement of CPSpyr$^-$, and an ACT$^-$ mutation will suppress the arginine requirement caused by a CPSarg$^-$ mutant (49, 60). The *ur-19* gene codes for DHO.

Attempts were made to estimate the molecular weights of CPSpyr and ACT and to establish whether they exist as a complex. Unfortunately, for technical reasons, accurate estimates of the molecular weights were not obtained. It was

observed that CPSpyr voids on Sephadex G-200, giving it a molecular weight in excess of 800,000, and most of the ACT activity was also voided. Elutions of wild-type and mutant extracts did confirm the existence of both CPSpyr and CPSarg, the latter eluting close to a catalase marker of 250,000 molecular weight, and hence well separated from CPSpyr (59).

PROTOZOA

Crithidia fasciculata

This parasitic flagellate protozoan is unusual in many ways regarding its intermediary metabolism, and one of these peculiarities is its arginine-pyrimidine system. Kidder et al. (77) first observed that the organism had an absolute requirement for arginine and that citrulline was a more effective pyrimidine precursor than was CO_2. Conversion of arginine to the pyrimidines was in three steps: arginine deiminase-catalyzed conversion to citrulline, thence to carbamoyl phosphate via citrullinase, and then ACT-catalyzed coupling of the carbamoyl phosphate with aspartate. OCT is absent, and CPS is only a minor source of carbamoyl phosphate. In later work, Kidder et al. (78) found no evidence of CPS activity in cell-free extracts. In vivo, it was observed that both CO_2 and the ureide carbon atom of citrulline were incorporated into pyrimidines.

As was seen with *L. leichmannii,* which also degrades arginine to carbamoyl phosphate, ACT is insensitive to feedback inhibition by pyrimidines. It exhibits hyperbolic kinetics with both substrates, having K_m values of 5 mM for aspartate and 0.5 mM for carbamoyl phosphate, although the kinetics are complicated by substrate inhibition by aspartate. ACT from *Crithidia* has a molecular weight of approximately 100,000 (78).

ANGIOSPERMS

Phaseolus aureus (Mung Bean)

There appears to be only one CPS enzyme in mung bean. It is inhibited by UMP and to a lesser extent by AMP, CMP, CTP, UTP, IMP, and GMP. It is activated by ornithine. It is glutamine dependent but can utilize ammonium ions with a lower affinity, the K_m values being 0.17 mM for glutamine and 6.1 mM for ammonium. The kinetics of CPS from mung bean are hyperbolic (116).

ACT is strongly inhibited by UMP, with UDP and UTP being progressively less effective. There is controversy as to the effect of other nucleotides. ACT shows hyperbolic kinetics with both substrates, the K_m being 0.09 mM for carbamoyl phosphate and 1.7 mM for L-aspartate. As was found for the pseudomonads, UTP converts a hyperbolic carbamoyl phosphate dependence curve to a sigmoidal one. ACT has two pH optima, at 8.0 and 10.2 (5, 115).

Estimates of the molecular weight of ACT are not in close agreement with each other, being at 83,000 and 128,000 (5, 115). Attempts have been made to separate UMP inhibition from the catalytic activity as with *E. coli.* Urea, *para*-chloromercuribenzoate, pH change, and heat treatment all give rise to a loss of activity (115). However, it is claimed that *para*-hydroxymercuribenzoate will desensitize ACT, and after such treatment, two new bands were found on polyacrylamide gel electrophoresis. The addition of 2-mercaptoethanol reversed the process, suggesting that ACT comprises two dissimilar subunits (5).

Pisum sativum (Garden Pea)

There appears to be one CPS enzyme, and this has been purified about 100-fold. It is highly unstable, but can be stabilized by its substrates, its activators, and such reagents as 2-mercaptoethanol and dithiothreitol (114).

The CPS is inhibited by uridine nucleotides, but not by those of cytidine. ADP and AMP also inhibit, although AMP is capable of activation, depending on the conditions used. L-Ornithine, which activates CPS from pea, opposes inhibition brought about by pyrimidines but increases inhibition by AMP or ADP. The other purines, GTP, GMP, ITP, and IMP, all activate (112, 114).

The enzyme has a higher affinity for L-glutamine than for ammonium ions. Its pH optimum is in the range of 8.1 to 8.2. It exhibits hyperbolic kinetics, having K_m values of 0.12 mM for glutamine and 0.39 mM for ATP (113).

Triticum aestivum (Wheat Germ)

There are no data on CPS in wheat.

ACT is inhibited by UMP and to a lesser extent by UDP. It exhibits very similar kinetics and a similar pH optimum to the ACT from *Phaseolus aureus* (161; R. J. Yon, Biochem. J. **121**:18P, 1971; Biochem. J. **124**:10P, 1971). Its molecular weight is 100,000, as judged by gel filtration (Yon, Biochem. J. **121**:18P, 1971). Electrophoresis through denaturing gels gave an estimate of its subunit molecular weight of 52,000, suggesting a dimeric protein. Molecular-weight determinations on nondenaturing gels gave a value of 97,000 in the presence of carbamoyl phosphate. In the presence of the inhibitor UTP, two bands were seen, one at 110,000 and one at 250,000. Since these various forms are interconvertible, the authors tentatively pro-

posed a dimer-hexamer equilibrium, which may explain why they found that UTP converted a hyperbolic substrate dependence to a sigmoidal one (J. E. Grayson and R. J. Yon, Biochem. Soc. Trans., in press).

METAZOA

Invertebrates (Excluding *Drosophila*)

The enzymes of carbamoyl phosphate synthesis and utilization have been investigated in a number of invertebrates. A glutamine-dependent CPS has been found in earthworm (*Lumbricus terrestris*), planarian (*Bipalium kewense*), and land snail (*Otala lactea*) mitochondria. This enzyme requires *N*-acetylglutamate, a precursor of ornithine, as an essential cofactor. Mitochondria also contain an ammonium-dependent, *N*-acetylglutamate-requiring CPS (148). The glutamine-dependent CPS has also been found in the mitochondria of a second land snail species, *Strophocheilus oblongus*, in which OCT activity has also been found. ACT activity, but very little CPS activity, was found in the cytoplasm (147). It is difficult to understand the function of these two CPS enzymes in invertebrates, but because of their location and substrates, both are presumably involved in the arginine biosynthetic pathway. A distinct possibility is that the ammonium-dependent enzyme is the glutamine-dependent enzyme minus its glutaminase subunit, lost during extraction, a problem encountered in *Neurospora* (R. H. Davis, personal communication). The existence of a pyrimidine-specific CPS remains unresolved.

Several parasitic invertebrates have been studied. No glutamine- or ammonium-dependent CPS has been found in the mitochondria or cytoplasm of the flatworms *Fasciola hepatica*, *Moneizia benedini*, and *Paramphistonium cervi* or the nematode *Ascaris suum* (81, 82). OCT is also absent, but ACT activity has been detected in the cytoplasm. As seen in *E. coli*, ACT activity is stimulated by ATP, but it differs from the situation in enteric bacteria by being insensitive to CTP. The pH optimum of 9.2 is much closer to that found in ACTs that are not subject to feedback inhibition, e.g., *Streptococcus faecalis* (82, 83). Although, in view of the instability of CPSs in many organisms, the failure to detect CPS activity cannot be taken as conclusive evidence for its absence, the absence also of OCT strongly suggests that these worms cannot synthesize arginine. This conclusion is consistent with these organisms' parasitic role, where exogenous arginine would be expected to be plentiful. It would be interesting to know whether exogenous arginine is the source of pyrimidines in these parasites as it is in *Crithidia* and *Lactobacillus*.

Drosophila melanogaster

The sex-linked *rudimentary* (*r*) mutants are pyrimidine auxotrophs (107). In addition, there are other pyrimidine auxotrophs mapping close to this region which give rise to normal wings, but in some cases female sterility, another aspect of the phenotype of *rudimentary*. It is therefore likely that all these pyrimidine auxotrophs are allelic (45, 46). Genetic studies of the *rudimentary* locus have yielded both recombination and complementation maps, and these are approximately colinear (25). The locus appears to specify CPSpyr, ACT, and DHO and has regions for each activity, albeit overlapping. The ACT-specific region is at one end of the gene, followed by the CPSpyr-specific region, with the DHO-specific region at the other end (68, 108, 131).

An enzyme complex with all three activities has recently been isolated and partially purified. It appears as two peaks on a sucrose density gradient, corresponding to 800,000 and 350,000 daltons (67). Söderholm et al. (139) found ACT activity at 390,000 daltons by gel filtration, which is presumably the same species as the lighter form mentioned above. A mutant without CPS activity had ACT with a molecular weight of 175,000, which may be an ACT subunit. The same authors also reported that ACT activity exhibits hyperbolic kinetics with both substrates, although this is complicated by substrate inhibition by aspartate (apparent K_m values of 5.4 mM for aspartate and 0.44 mM for carbamoyl phosphate). ACT is not inhibited by pyrimidines, a feature observed with most of these enzyme complexes.

Rana catesbeiana (Bullfrog)

CPS, OCT, and other enzymes of the arginine biosynthetic pathway have been detected in a number of amphibia (24). In *R. catesbeiana* there are two CPS enyzmes, one of which appears specific for the arginine biosynthetic pathway, utilizes ammonium ions as a nitrogen source, and requires *N*-acetylglutamate as a cofactor (48, 69), and the other is pyrimidine-specific and utilizes glutamine (86).

The arginine-specific CPS from frog liver has K_m values of 1, 3 to 9, and 3 mM for ammonium, bicarbonate, and ATP, respectively. It has a pH optimum of 7.2 to 7.4 (69), and its molecular weight as determined by ultracentrifugation is approximately 315,000 (96).

The pyrimidine-specific CPS from the ovary of *R. catesbeiana* has been shown to exist in a

complex with ACT and DHO, with a molecular weight of approximately 900,000 as shown by their cosedimentation on a glycerol gradient. This complex is capable of dissociating to smaller species if the concentration of various effectors (ATP, UTP, Mg^{2+}, PRPP, and inorganic phosphate [P_i]) are altered. CPS and DHO can both sediment at a size of 260,000 to 280,000 daltons, possibly as separate peaks. Depending on the exact conditions, ACT sometimes appears to be in equilibrium between several different-size species, and at other times forms a discrete peak at 110,000 daltons. These molecular-weight changes may be partially reversible, since much of the three activities, when separated and then recentrifuged together on a fresh glycerol gradient, reassociate to form a complex similar, but not identical, to the original enzyme complex (75).

ACT and DHO activities are unaffected by ATP, CTP, UTP, PRPP, P_i, or Mg^{2+}, whereas the glutamine-dependent CPS is inhibited by UTP and activated by PRPP, P_i, and Mg^{2+}. This pyrimidine-specific CPS can use either ammonium or glutamine as nitrogen source, but has an affinity for the latter substrate three orders of magnitude greater than for ammonium. The glutamine-dependent CPS from eggs of *Rana pipiens* has very similar properties (75, 86).

Mammalian Systems

A number of different species and different tissues have been investigated, and although they have many features of pyrimidine metabolism in common, there are some differences which may reflect tissue-specific or species-specific properties.

Two main CPS enzymes have been described. CPS-I is mitochondrial and is found, in conjunction with OCT, in high levels in the liver. It uses ammonium ions as an amino source and requires *N*-acetylglutamate as a cofactor. CPS-II is cytoplasmic and is associated with ACT, and the CPS-II and ACT activities parallel each other. This second system is particularly abundant in neoplastic cells. CPS-II utilizes glutamine as an amino source. Because of these considerations, it is likely that CPS-I is arginine specific and CPS-II is pyrimidine specific (71), but there is some controversy on this point. CPS-I from rat liver is moderately inhibited by all pyrimidines, particularly CTP (76). However, CPS-II from rat liver, mouse spleen, or mouse tumor cells is powerfully inhibited by UTP and activated by PRPP (98a, 138, 146). In vitro experiments with intact isolated mitochondria have shown that carbamoyl phosphate produced by CPS-I is able to traverse the double membrane of the mitochondria to become available to *E. coli* ACT in the reaction mixture (102, 103). This is unlikely to occur in vivo since the intramitochondrial OCT is at a very much higher level than the extramitochondrial ACT (71).

A third CPS type has been isolated from lactating bovine mammary tissue. This enzyme uses ammonium ions, like CPS-I, but does not require *N*-acetylglutamate. It is activated by PRPP and inhibited by pyrimidines, but with a different specificity from CPS-II (151).

CPS-I has been investigated in rat liver. It displays hyperbolic kinetics for all its substrates, although ATP sometimes exhibits sigmoidal kinetics. Elliott and Tipton (43) have suggested that this might be due to a failure to keep the free Mg^{2+} concentration constant in the experimental procedure. CPS-I is unable to use glutamine as the amino source, and K_m values for HCO_3^-, NH_4^+, and ATP are in the ranges 4 to 5, 1 to 2, and 0.5 to 3 mM, respectively (76). CPS-I from bovine liver has essentially similar K_m values (43). Centrifugation of purified CPS-I from rat liver on a sucrose density gradient revealed an equilibrium between two molecular-weight forms at 316,000 and 160,000 (152).

CPS-II from mouse spleen and rat liver exhibits sigmoidal kinetics with ATP (99, 145). The enzymes from mouse spleen, Ehrlich ascites cells, and rat liver all show hyperbolic kinetics with the other substrates. For mouse spleen, K_m values for glutamine, NH_4^+, and HCO_3^- were reported as 0.013, 15, and 9.5 mM, respectively, with values for the enzyme from ascites cells of 0.01 mM for glutamine and 5 mM for NH_4^+. The pH optimum is approximately 7.4 (56, 57, 145). CPS-II from all these sources is highly labile, but has been stabilized by glycerol, dimethyl sulfoxide, dithiothreitol, and glutamine (65, 98a, 138, 144).

ACT has been studied in a wide variety of mammalian tissues, and in no case has it been observed to be inhibited by any pyrimidine (22, 31, 61, 138).

CPS-II and ACT have now been found to be associated with the third enzyme in the pyrimidine biosynthetic pathway, DHO in an enzyme complex (29, 98a, 99, 100, 137, 138). Earlier work on different mammalian tissues revealed a large range of molecular weights for these activities. Koskimies et al. (80) found ACT activity from various mouse and rat tissues and from HeLa cells in two forms, 900,000 and 600,000 daltons. Proteolysis of these extracts gave rise to a third form of ACT, at 80,000 daltons. Unfortunately CPS and DHO activities were not measured. Ito and Uchino (66) purified a CPS-ACT complex

from human lymphocytes and measured its molecular weight as 600,000. No estimate of DHO on this preparation was reported. Shoaf and Jones (138) estimated the molecular weight of the CPS-ACT-DHO complex from mouse ascites cells by sucrose gradient centrifugation and found it to be in the range of 800,000 to 850,000. If the concentration of the stabilizer dimethyl sulfoxide was lowered, three peaks were obtained on the gradient: the CPS activity was at 150,000 to 200,000, and the ACT and DHO cosedimented in two peaks at 400,000 to 450,000 and 650,000 to 700,000. Mori et al. (98a) performed similar experiments on the purified complex from rat liver. In the presence of ATP, all three activities cosedimented at 1,090,000 daltons. If ATP was removed, two peaks were observed at 680,000 and 220,000 daltons, both possessing CPS activity. ACT and DHO were found to cosediment with the larger form, although there was a suspicion that their peak might correspond to a slightly lower molecular weight than the 680,000 CPS. More recently, Mori and Tatibana (100) estimated the molecular weight of the CPS-ACT-DHO complex from rat ascites cells by ultracentrifugation and found it to be 870,000. Electrophoresis in the presence of SDS gave a subunit size of 210,000. Similar results were obtained for cultured Chinese hamster cells (29). These latter workers then estimated the molecular weight of the entire complex by the same method after first chemically cross-linking the subunits to prevent dissociation. Using this method of pretreatment and gel electrophoresis, they observed many bands corresponding to integral multiples of the monomer up to a dodecamer. There were two major bands, which corresponded to a hexamer (1,200,000 daltons) and a trimer (600,000). Electrophoresis under nondenaturing conditions gave two bands, and ultracentrifugation gave two peaks, in each case consistent with the trimer and hexamer and strongly suggesting that the native enzyme can exist as either of these oligomers. The same workers also estimated the number of ACT catalytic sites by titration with the aspartate analogue *N*-phosphonoacetyl-L-aspartate, which they found bound to the complex in the ratio of 1 molecule of *N*-phosphonoacetyl-L-aspartate per 230,000 daltons. These data are taken as evidence for a hexameric protein, in equilibrium with a trimeric form, with subunits of molecular weight 200,000, each of which catalyzes all three shapes.

Since the work of Coleman et al. (29) utilized SDS-gel electrophoresis to determine the molecular weights of both subunit and native enzyme, it is the most reliable. Their hexameric model can also be reconciled with the earlier data, since the various CPS and ACT-DHO species reported by Shoaf and Jones (138) and Mori et al. (98a) could well be proteolytic fragments, an interpretation consistent with the failure of either of these systems to show reassociation of the forms back to the native enzyme. It is, however, just possible that the enzyme complex is capable of existing in different quaternary structures under different conditions of extraction and purification. Thus, for example, the "native" enzyme of Shoaf and Jones could be a tetramer. However, it is difficult to see why their species of 400,000 to 450,000 daltons, which on this model would be a dimer, should lack CPS activity, whereas the 150,000- to 200,000-dalton form, which would be the monomer, possesses CPS activity.

Avian Systems

It has proved very difficult to demonstrate any CPS activity in birds (i.e., pigeon and chicken) to date. Schulman and Badger (M. P. Schulman and S. J. Badger, Fed. Proc. **13**:292, 1954) followed the fate of intraperitoneally injected ureido ^{14}C-labeled citrulline in the former and showed that pyrimidines were very efficiently labeled whereas purines and carbon dioxide were not. Furthermore, the pyrimidines were labeled in the C-2 position, which is the carbon atom normally derived from HCO_3^-. This strongly suggested that a similar pathway to that found in *Lactobacillus* was operating. However, Bowers and Grisolia (20) were unable to demonstrate any labeling of carbamoyl aspartate after in vitro administration of labeled CO_2, citrulline, or arginine to lysed pigeon liver homogenates. This difference may of course be due to a labile enzyme which is inactive in vitro. In vivo administration of ^{14}C-labeled carbonate to chickens led to extensive labeling of the C-2 atom of pyrimidines isolated from the liver, suggesting the involvement of a CPS (133). However, although she was able to demonstrate ACT, DHO, OMP pyrophosphorylase, and OMP decarboxylase activities, no CPS activity could be detected.

Hager and Jones (57) found low levels of glutamine-dependent CPS in the soluble fraction of pigeon liver extracts and even lower levels from chicken liver. They also found ACT activity in the soluble fraction, and in both cases this was at a much lower level than that found in mammalian tissues, strongly suggesting that the evidence for this avian CPS was real.

Further evidence for a pyrimidine-directed CPS activity came from labeling of minced tissue of estrogen-stimulated chick oviduct using the ^{14}C-labeled precursors bicarbonate, carbamoyl

phosphate, and carbamoyl aspartate. In all three cases, labeled orotate could be isolated. However, if uridine, adenosine, or guanosine was present during the incubation, the labeling from bicarbonate, but not from carbamoyl phosphate or carbamoyl aspartate, was markedly inhibited. Very similar results have been obtained in a mammalian system under hormonal stimulation, that of the lactating rat mammary gland (54).

ACT activity has not been difficult to detect in either pigeon or chicken tissues. It has been shown to exist in two molecular-weight forms in chicken liver, intestine, and embryo, at 900,000 and 600,000 (80). These authors obtained identical molecular weights also with a variety of rat and mouse tissues (see "Mammalian Systems").

It would appear, therefore, that a de novo pyrimidine biosynthetic pathway exists in birds similar to the one found in mammals. However, under certain conditions an alternative pathway via citrulline and carbamoyl phosphate appears to operate, possibly to different extents in different tissues. This would explain the much lower activity of avian, as opposed to mammalian, CPS and ACT. It would also explain the observation that chick tissues require arginine to be supplied, and that citrulline but not ornithine would satisfy this requirement (143). However, these workers reported the presence of the later enzymes of the arginine biosynthetic pathway (OCT, argininosuccinate synthase, and argininosuccinase) and also arginase in chick kidney, although they found no CPS activity. None of these activities could be demonstrated in chick liver.

Until an arginine-specific CPS is shown to exist, it remains uncertain whether birds have the potential for arginine biosynthesis even if, for much of the time, dietary arginine switches off such a pathway.

CONCLUSIONS

Three main classes of organization of the initial steps of pyrimidine biosynthesis have so far been identified. Interestingly, examples of each have been found among both procaryotes and eucaryotes. Higher plants such as pea and mung bean and certain bacteria such as *Escherichia coli, Salmonella typhimurium,* and the pseudomonads all appear to rely on a single CPS enzyme to provide a common pool of carbamoyl phosphate for both pyrimidine and arginine biosynthesis. We shall refer to this type of organization as class I. Class II organization is found in most animals (both vertebrates and invertebrates), fungi, and the procaryote *Bacillus subtilis* and involves two CPS enzymes, one for each pathway. Among the eucaryotes in this class, the two carbamoyl phosphate pools are normally kept physically separate from each other. Organisms with an absolute requirement for arginine comprise class III, in which there is a total lack of CPS activity, the carbamoyl phosphate being produced by the degradation of exogenously supplied arginine or citrulline. Examples of class III organization are *Lactobacillus leuchmannii, Streptococcus faecalis,* and *Aeromonas salmonicida* among the procaryotes and the protozoan parasite *Crithidia,* plus probably nematodes and flatworms among the eucaryotes.

These common forms of organization of this particular aspect of metabolism among otherwise very different organisms are almost certainly the result of convergent evolution. This is particularly so for the class III pathway, where organisms of this type are parasitic, inhabiting nutritionally very rich environments. In such an environment, all were probably under similar selective pressure to lose the primary biosynthetic pathway to carbamoyl phosphate, replacing CO_2 as a precursor with more readily available arginine.

Since all higher animals are included in class II, one might assume that the class is evolutionarily advanced. Within the class, there seems to be a steady evolutionary progression in favor of clustering of the genes controlling the early steps in pyrimidine biosynthesis and their associated enzyme activities. The first step appears to have been the duplication of the CPS gene and enzyme and the specialization of one for arginine synthesis and the other for pyrimidine synthesis. This condition is found in bacteria such as *B. subtilis.* This duplication permitted the coupling, genetically and biochemically, of the pyrimidine-specific CPS with the next enzyme in the pathway, ACT, in the situation found in all fungi. The genetic cause would appear to be a translocation resulting in gene fusion, giving the bifunctional gene seen in the fungi, or trifunctional if one differentiates between the glutaminase function of the CPS and CPS activity proper. The resultant gene fusion parallels the complexing of the enzyme activities of the gene products, resulting in the advantages of both coordinate regulation and more efficient channeling of the carbamoyl phosphate in the pyrimidine pathway. In *Drosophila* and other nonparasitic invertebrates, the third enzyme in the pyrimidine biosynthetic pathway, DHO, is included in both the gene complex and the enzyme complex, and in the vertebrates, although it is not known whether such a gene complex exists, the enzyme complex of pyrimidine-specific CPS, ACT, and DHO certainly does.

Although it is possible to divide organisms into three major classes on the basis of their

pyrimidine metabolism, considerable variation exists within each class. The CPS enzymes from class I, as far as is known, are all inhibited by UMP, activated by ornithine, and unaffected by *N*-acetylglutamate. They all have a higher affinity for glutamine than ammonia as nitrogen source. Their ACT enzymes are all inhibited by pyrimidines, but relative sensitivities vary greatly. In pseudomonads and angiosperms, ACT shows similar hyperbolic kinetics for both substrates, converting to sigmoidal in the presence of UTP. ACT kinetics of *E. coli* and *S. typhimurium* are sigmoidal for both substrates in the presence and absence of the inhibitor CTP.

In class II, with the possible exceptions of *B. subtilis* and *S. cerevisiae,* where channeling of carbamoyl phosphate is not very efficient, the first unique step of the pyrimidine biosynthetic pathway is catalyzed by CPSpyr. This is reflected in the lack of feedback inhibition by pyrimidines in all class II organisms except

TABLE 1. *Summary of pyrimidine genes and enzymes*

Organism	No. of CPS enzymes	Gene complex	Enzyme complex	ACT inhibition by pyrimidines	ACT kinetics[a]	Class
Escherichia coli	1	No	No	Yes	Both sig.	I
Salmonella typhimurium	1	No	No	Yes	Both sig.	I
Bacillus subtilis	2	CPS-ACT-DHO?	?	No	Both hyper.	II
Lactobacillus leichmannii	0			No	Both hyper.	III
Streptococcus faecalis	0			No	Both hyper.[b]	III
Pseudomonas spp.	1	No	No	Yes	Both hyper.[c]	I
Aeromonas salmonicida	0			?	?	III
Citrobacter freundii	?	?	?	Yes	Both hyper.	I?
Serratia marcescens	1 or 2	?	?	Yes?	?	I?
Halobacterium cutirubrum	?	?	?	Yes	Sig. with aspartate	I?
Saccharomyces cerevisiae	2	CPS-ACT	CPS-ACT	Yes	Both hyper.	II
Aspergillus nidulans	2	CPS-ACT	CPS-ACT	?	?	II
Neurospora crassa	2	CPS-ACT	CPS-ACT	No	Both hyper.	II
Coprinus radiatus	2	CPS-ACT	CPS-ACT	?	?	II
Crithidia fasciculata	0			No	Both hyper.	III
Phaseolus aureus	1	?	No?	Yes	Both hyper.[c]	I
Pisum sativum	1	?	?	?	?	I
Triticum aestivum	?	?	No?	Yes	Both hyper.[c]	I
Drosophila melanogaster	2?	CPS-ACT-DHO	CPS-ACT-DHO	No	Both hyper.	II
Parasitic flatworms	0			No	?	III
Parasitic nematodes	0			No	?	III
Rana catesbeiana	2	?	CPS-ACT-DHO	No	?	II
Birds	2?	?	CPS-ACT-DHO	?	?	II?
Mammals	2 (3?)	?	CPS-ACT-DHO	No	Both hyper.	II

[a] Sig., Sigmoidal; hyper., hyperbolic.
[b] Hyperbolic at high anion concentration.
[c] Hyperbolic, but converted to sigmoidal kinetics for carbamoyl phosphate by UTP.

yeast. In this class, as stated above, CPSpyr exists in complex with ACT, and in animals also with DHO. There appear to be large differences in kinetics of the three activities within class II.

There are few data on ACT enzymes from class III organisms. The enzymes from *Crithidia,* parasitic nematodes and flatworms, *L. leichmannii,* and *S. faecalis* are all insensitive to pyrimidines, which is consistent with ACT not catalyzing the first step in the pyrimidine pathway. In *L. leichmannii* but not in *A. salmonicida,* the enzyme which does catalyze the first step, arginine deiminase, is inhibited by TMP. The arginine dihydrolase enzyme system used by these organisms is probably also used by birds under certain circumstances. However, since birds possess at least the pyrimidine-specific CPS, probably in a complex with ACT and DHO, they have been included in class II.

Thus far, we have not categorized the bacteria *Serratia marcescens, Citrobacter freundii,* and *Halobacterium cutirubrum* due to lack of data on CPS. However, all three have ACT enzymes subject to inhibition by pyrimidines, although with *S. marcescens* there is some disagreement on this point. Furthermore, as already mentioned, some CPS activity has been reported for *S. marcescens.* There would therefore be some justification for tentatively assigning these bacteria to class I.

Our conclusions are summarized in Table 1.

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